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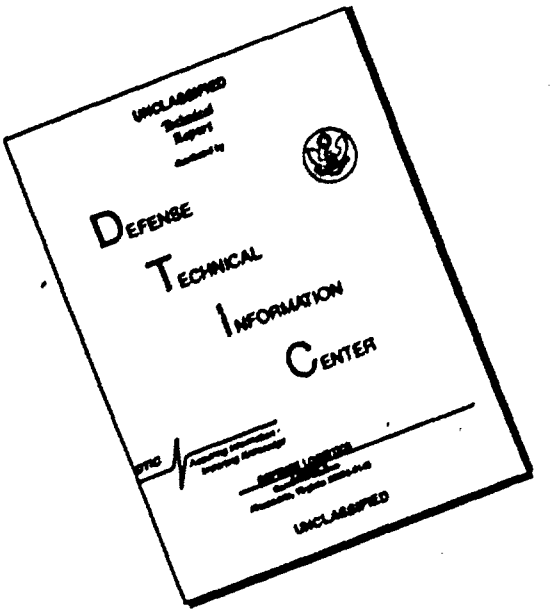

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DEMONSTRATION OF THE ORIGIN OF HUMAN MAST CELLS FROM CD34+ BONE MARROW PROGENITOR CELLS

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It has been established that murine mast cells are derived from a pluripotent bone marrow stem cell. In humans, the corresponding pluripotent cell is included in the CD34+ bone marrow population. To determine whether human mast cells arise from CD34+ human progenitor cells, enriched CD34+ cells were cultured over agarose surfaces (interphase cultures) or cocultured with mouse 3T3 fibroblasts in the presence of recombinant human (rh) IL-3. The presence of both mast cells and basophils was determined using a variety of histochemical and immunohistologic techniques, including immunogold labeling for IgE receptors and mast cell tryptase. Mast cells and basophils continued to appear in cultures when T cell, B cell, macrophage, and eosinophil committed progenitor cells were removed, but were not seen in cultures from which CD34+ cells were removed. CD34+ cells layered over agarose in the presence of rhIL-3 were shown to give rise to cultures that contained mast cells (1 to 5%) and basophils (25 to 40%). Cultures supplemented with rhIL-4 showed no additional increase in mast cells or basophils. CD34+ cells cocultured with 3T3 fibroblasts in the presence of rhIL-3 gave rise to mast cells within the fibroblast monolayer, which by 6 wk comprised up to 46% of the monolayer. CD34- cells on 3T3 fibroblasts gave rise to few mast cells (2% of the monolayer). Mast cell granules from interphase cultures contained homogeneous electron-dense material. In contrast, mast cells within 3T3 monolayers at 6 wk contained a variety of granule morphologies, including scroll, mixed, reticular, dense core, or homogeneous patterns. We conclude that both human mast cells and basophils arise from CD34+ human progenitor cells.

In the mouse, mast cell progenitor cells have been shown to originate in bone marrow and form colonies (CFU-S) in mouse spleen (1–3). These mast cell progenitor cells selectively adhere to mouse embryonic skin monolayers (4, 5), express mRNA for the subunits of IgE receptors before phenotypic differentiation (6, 7), and proliferate in the presence of IL-3 and IL-4 (8, 9).

In humans, mast cells and basophils have been cultured from bone marrow in agarose interphase cultures (10, 11). Basophils stained metachromatically and had surface IgE receptors (12), as did mast cells, but lacked tryptase in the granules. The cell of origin in human marrow giving rise to basophils is believed to be the CD34+ human progenitor cell, since CD34+ cells cultured in liquid suspension cultures or as colonies in methylcellulose give rise to basophils in addition to other cell lineages (13–16). Studies to date, however, have not established the origin of human mast cells from CD34+ cells and, therefore, the relationship of CD34+ cells to mast cells has remained speculative.

Recently, it was shown that human cord blood mononuclear cells cocultured with mouse 3T3 fibroblasts give rise to tryptase-positive cells with granule ultrastructure characteristic of mast cells (17). To determine if CD34+ cells give rise to mast cells, cultures depleted of T cell, B cell, macrophage, or eosinophil progenitor cells, cultures of highly purified (>99% purity) CD34+ cells, and cultures of CD34- cells were examined to determine if these cultures gave rise to mast cells. As will be demonstrated, CD34+ cells, and not those committed progenitor cells of the T cell, B cell, macrophage, or eosinophil lineage, give rise to both mast cells and basophils. Cocultures of CD34+ cells with 3T3 fibroblasts gives rise to mast cells with a variety of granular morphologies more reminiscent of mature mast cells.

MATERIALS AND METHODS

Materials. SeaKem LE agarose (FMC BioProducts, Rockland, ME), 2-ME, Fast Blue B salt (Sigma Chemical Co., St. Louis, MO), monoclonal anti-Leu-5b (CD2), anti-Leu-12 (CD19), anti-Leu-16 (CD20), anti-Leu-M3 (CD14), anti-HPCA-1 (Becton Dickinson, Mountain View, CA), penicillin-streptomycin, L-glutamine, sodium pyruvate, and nonessential amino acids (Flow Laboratories, McLean, VA), heat-inactivated FCS (Grand Island Biological Co., Long Island, NY), high glucose, sodium pyruvate supplemented DMEM[2] (Irvine Biologicals, Irvine, CA), heat-inactivated bovine serum (Hyclone, Logan, UT), rhIL-3 (5,000 U/ml and 1.4×10^7 U/ml) and rhIL-4 (20,000 U/ml) (Genzyme, Boston, MA), Ficoll-Paque (Pharmacia, Piscataway, NJ), 50-mm tissue culture flasks, 16-mm 24-well tissue culture plates, cell scrapers (Costar, Cambridge, MA), 100 mm × 15 mm Petri plates (no. 1029), 35-mm culture wells (Falcon, Cockeysville,

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[2] Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle's medium; complete 1640, RPMI 1640 supplemented 4 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids; complete DMEM, Dulbecco's modified Eagle's medium with high glucose, sodium pyruvate and 10% bovine serum; rh, recombinant human.

MD), rabbit complement (Cedarlane Laboratories, Ontario, Canada), and 3T3 Swiss albino fibroblasts (American Type Tissue Culture, Rockville, MD) were obtained from the sources indicated. All other materials were of reagent grade and were obtained commercially.

Preparation of soft agarose. A soft agarose culture system using a single layer of agarose overlaid with a suspension of cells was prepared in 24-well plates (10, 11). The agarose layer in each well consisted of 0.63 ml composed of 0.32 ml of complete 1640, 0.09 ml of 2× concentrated RPMI 1640, 0.13 ml of heat-inactivated FCS, and 0.09 ml of a 3.3% (w/v) stock agarose solution in distilled water. In experiments performed using rhIL-3 alone or in combination with rhIL-4, recombinant factors were added to complete 1640 to give the appropriate final concentrations. The agarose was allowed to equilibrate at 37°C in 5% CO_2 for 1 h before use.

Bone marrow cultures. Human bone marrow cells were obtained from iliac crest aspirates of patients under evaluation for mastocytosis following informed consent. Cells were collected into 10-ml plastic syringes containing 1 ml of preservative-free heparin (1000 U/ml), diluted 1:6 in complete 1640, layered over Ficoll-Paque, and centrifuged at 800 × *g* for 20 min. The mononuclear cell layer was harvested and washed four times with complete RPMI 1640 medium containing 1% heat-inactivated FCS. Cells (2×10^5) were resuspended in 400 μl of complete 1640 with 10% heat-inactivated FCS and appropriate concentrations of rhIL-3 alone or in combination with rhIL-4 and layered over agarose in each well. Cells within each agarose-containing well were fed weekly by removing approximately 200 μl of the fluid over the agarose and replacing it with complete 1640 containing 10% heat-inactivated FCS and appropriate concentrations of recombinant factors. Cells were harvested by gently pipetting culture media several times over the agarose surface. Total cell counts were determined weekly. Mast cell and basophil numbers were determined on Wright-Giemsa stained cytopreparations (Shandon, Pittsburgh, PA) on the basis of morphology. These cell counts were confirmed by staining sequential slides with acid toluidine blue for basophils and mast cells and for human mast cell tryptase. Cell counts never differed by more than 5% and thus, routine cell counts determined by Wright-Giemsa-stained sections were used in data analysis.

Histochemical stains. Wright-Giemsa cell staining was completed using an automated slide stainer (Hematek; Miles Laboratories, Mishawaka, IN). Toluidine blue staining (pH 4.5), which stains mature connective tissue mast cells but not mucosal mast cells or basophils, and acidic toluidine blue (pH 1.0), which stains both basophils and all mast cells, was performed as described on slides fixed in Mota's lead acetate (18–20).

Anti-tryptase staining. Qualitative tryptase enzyme determinations of cytocentrifuged cell preparations were performed as described (21, 22). Briefly, slide preparations were placed in Carnoy's fixative for 15 min, rinsed, and exposed to goat serum for 2 h at room temperature. The slides were incubated overnight at 4°C with monoclonal mouse anti-human tryptase (G3) (1.76 μg/ml; a gift of L. B. Schwartz, Medical College of Virginia, Richmond, VA), washed, and incubated with a 1:50 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h at room temperature. Slides were developed for 8 to 10 min in a freshly prepared solution of fast blue Roussin's red (1 mg/ml) in 0.1 M Tris HCl, pH 8.2, containing naphthol AS-MX phosphate (0.2 mg/ml; 10 mg of naphthol AS-MX phosphate per ml dimethylformamide stock). Anti-tryptase positive mast cells stained blue. Results were expressed as the percentage of cells staining positively for tryptase.

Selective adherence (panning) of cells. Cells expressing T cell (CD2), B cell (CD19 and CD20), eosinophil, or macrophage (CD14) cell surface markers were removed by adherence as described (13). Briefly, Falcon 1029 culture plates were incubated overnight at 4°C with 100 μg/ml goat anti-mouse IgG in 10 ml of PBS containing 0.1% BSA, pH 9.5. Bone marrow cells (10×10^6) were incubated with either anti-CD2, anti-CD19 and -CD20, eosinophil receptor antibody, 4A17 (a gift of Dr. Tom Nutman, National Institute of Allergies and Infectious Diseases, National Institutes of Health), or anti-CD14 for 1 h at 4°C and then washed twice with PBS. Antibody-treated cells were then suspended in 6 ml of buffer, poured onto coated plates, and allowed to incubate for 2 h at 4°C with gentle mixing. Nonadherent cells were removed, washed twice, and allowed to adhere a second time. Nonadherent cells were then resuspended in complete media with 10% heat-inactivated FCS and incubated with a 1:5 dilution of rabbit C for 1 h at 37°C. Cells remaining after lysis were washed, resuspended in medium [illegible] and cultured over agarose surfaces. In all experiments, flow microfluorometric analysis of bone marrow cells before adherence and after lysis was performed to verify the removal of the CD2, CD19 and CD20, 4A17, or CD14 positive cells.

Purification of CD34⁺ hemopoietic progenitor cells. Cells expressing the CD34 hemopoietic progenitor cell Ag were isolated from bone marrow mononuclear cells by panning in initial studies, and in later studies by positive immunomagnetic selection. For enrichment by panning, cells were incubated with mAb My10 (anti-HPCA-1) and allowed to attach to plates coated with goat anti-mouse IgG (23). Plates were gently rinsed four times, and adherent cells were removed with a cell scraper. For positive immunomagnetic selection, CD34⁺ cells in the mononuclear cell suspension were rosetted with the high avidity CD34 mAb K6.1 (24) linked to magnetic Dynabeads, and were separated by attraction to a rare earth magnet. After three to four successive selections, the beads were detached from the CD34⁺ cells (within 2 h) and removed magnetically (25) (S. W. Kessler, manuscript in preparation). In other studies, CD34⁺ cells recovered by this immunomagnetic procedure have been shown to be unaltered phenotypically with respect to expression of surface membrane Ag, including CD34 (25). Upon restaining with My10 antibody (HPCA-1) and isotype-specific secondary antibody, and flow microfluorometric analysis, cells were consistently >99% CD34⁺. In all experiments, recovered cells were cultured over agarose surfaces in the presence or absence of rhIL-3, or in combination with rhIL-4, or cocultured with mouse 3T3 fibroblasts.

Coculture of CD34⁺ cells with 3T3 fibroblasts. A modification of a previously described method was used in the coculture of highly enriched CD34⁺ human progenitor cells with Swiss albino mouse 3T3 fibroblasts (26–28). Briefly, 3T3 fibroblasts, suspended in DMEM supplemented with 10% bovine serum (complete DMEM), were allowed to grow to confluency as a monolayer on 50-mm^2 plastic flasks or 35-mm^2 plastic well surfaces. Approximately 1 to 2×10^6 CD34⁺ cells per ml complete DMEM were layered over 3T3 monolayers and allowed to incubate for 72 h. Nonadherent cells were poured off, and the remaining adherent cells were cultured in complete DMEM containing 200 U/ml rhIL-3. IgE_{PS} (gift of Henry Metzger, M.D., National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health) was added to the media at a concentration of 5 μg/ml, since murine IgE has been shown to augment the proliferation of IL-3-induced murine mast cells in the presence of IL-3 (29). Half of the culture media were removed weekly and replaced with complete DMEM supplemented with appropriate concentrations of rhIL-3 and IgE_{PS}. Cultures were harvested at 6- to 8-wk intervals and prepared for electron microscopy.

Electron microscopy and immunohistochemistry. Cell preparations were labeled first for the presence of IgE receptors and subsequently prepared for electron microscopy and tryptase labeling. Thus, cells in interphase cultures were harvested, resuspended, and centrifuged twice with PBS containing 0.1% BSA. Cells were incubated with 10 μg/ml human IgE_{PS} for 30 min at 37°C, with control cells incubated in buffer alone. The cells were next centrifuged and incubated with goat anti-human IgG conjugated to 10-nm gold particles for 30 min at room temperature. Cell suspensions were then fixed in a mixture of 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 3 h at room temperature. Following centrifugation with cacodylate buffer, the cells were suspended in warm 2% agarose and centrifuged. Cell pellets were post-fixed 1 h at room temperature in cacodylate buffered 1% OsO_4, dehydrated in a graded ethanol series, and embedded in EM bed 812/Araldite 502. Thin sections were cut and mounted on uncoated nickel grids. For tryptase labeling, sections on grids were placed on a 0.56 M (saturated) aqueous solution of sodium metaperiodate for 70 min at room temperature, rinsed with PBS, and incubated with either normal goat serum or goat IgG (0.5 mg/ml) 1 h at room temperature. Grids were incubated overnight at 4°C with antitryptase (G3) (48 μg/ml) in PBS containing 0.1% BSA. Sections were washed 5 min each with 0.5 M Tris, pH 7.2, Tris-buffered saline, and Tris containing 0.2% BSA. Sections were placed on Tris containing 1% BSA, pH 8.2, for 5 min followed by goat anti-mouse IgG conjugated to 5-nm gold particles for 1 h at room temperature. Sections were washed 5 min each in Tris containing 0.2% BSA, Tris-buffered saline, and water. Controls included incubation with preimmune IgG and omission of the primary antibody. Sections were stained for 15 min with aqueous uranyl acetate and examined with a Phillips 300 electron microscope.

Cocultured CD34⁺ and 3T3 cells were labeled and processed in situ to preserve the relationship between adherent mast cells and fibroblasts. Labeling and processing of cocultured cells was as described for interphase cultures. Briefly, 0.5-μm-thick sections were cut and stained with acid toluidine blue. Sections containing cells that stained metachromotically were thin sectioned and labeled for tryptase and examined by electron microscopy.

RESULTS

As shown previously (10, 11), mast cells and basophils developed from human bone marrow cells cultured over

agar or agarose surfaces in the presence of rhIL-3. To determine whether basophils or mast cell numbers in culture were affected by the removal of other committed progenitor cells, T cells, B cells, and macrophages were selectively removed using mAb to CD2, CD19 and CD20, and CD14, respectively, and complement lysis. Eosinophils were removed using the mAb 4A17 and complement lysis. Remaining cells were then cultured over agarose surfaces in the presence of rhIL-3 and examined weekly. Cytocentrifuged preparations of cultured cells were stained with Wright-Giemsa and toluidine blue, and were examined for the presence of tryptase-positive cells. Mast cells and basophil numbers were not significantly affected by the removal of committed T cells, B cells, macrophages, or eosinophils (Fig. 1, *A* and *B*). Mast cells and basophils were effectively removed from culture, however, when $CD34^+$ ($My\text{-}10^+$) progenitor cells were depleted from bone marrow cells by panning (Fig. 2, *A* and *B*). The remaining $CD34^-$ cells differentiated predominantly into macrophages with no appreciable increase in total cell number.

To decrease the contaminating cells in the progenitor cell population and determine the extent of growth of mast cells and basophils from $CD34^+$ cells, highly purified preparations of $CD34^+$ progenitor cells (>99% $CD34^+$) were obtained using immunomagnetic positive selection. Cells purified in this manner were detached from magnetic spheres and cultured over agarose surfaces. Mast cells averaged 1 to 5% and basophils averaged 25 to 40% of total cell numbers. Mast cells appeared morphologically identical to those cells obtained in earlier experiments with unenriched and enriched panned bone marrow preparations (Fig. 3) and peaked in number by 2 to 3 wk (Fig. 4, *A* and *B*). The addition of rhIL-4 500 U/ml did not increase basophil and mast cell numbers over those obtained with rhIL-3 100 U/ml alone. Cells were stained for the presence of surface IgE receptors and tryptase within granules using 10 nm and 5 nm size gold spheres, respectively. In all cultures, basophils had smooth surfaces bearing IgE receptors and granules containing dense flocculent material but no tryptase (Fig. 5). Agarose-cultured mast cells had surfaces with cytoplasmic extensions and stained positive for IgE receptors (Fig. 6). Mast cell granules contained homogeneous electron-dense material and stained positive for tryptase (Fig. 6, *inset*).

To further examine whether mast cells arise from $CD34^+$ cells, $CD34^+$ cells were next cocultured with 3T3 fibroblasts, known to permit mast cell differentiation (17). After 6 wk in culture in the presence of rhIL-3 and IgE_{PS}, adherent $CD34^+$ cells gave rise to metachromatically staining cells resembling tissue mast cells. Approximately 46% of cocultured $CD34^+$ cells were toluidine blue positive after 6 wk in culture, whereas approximately 2% of cocultured $CD34^-$ cells stained positive. Mast cells in $CD34^+$ cocultures were spindle or oval shaped, had irregular surfaces with cytoplasmic extensions, and contained

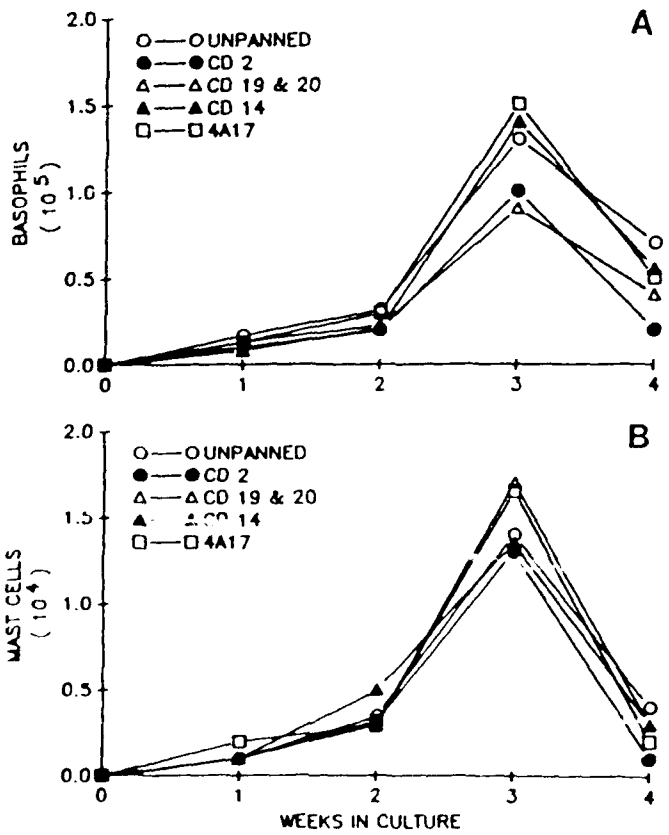

Figure 1. Growth of human basophils (*A*) and mast cells (*B*) after selective depletion of T cell, B cell, macrophage, or eosinophil committed precursors. Basophil and mast cell numbers were unaffected by removal of precursors. Average of two experiments.

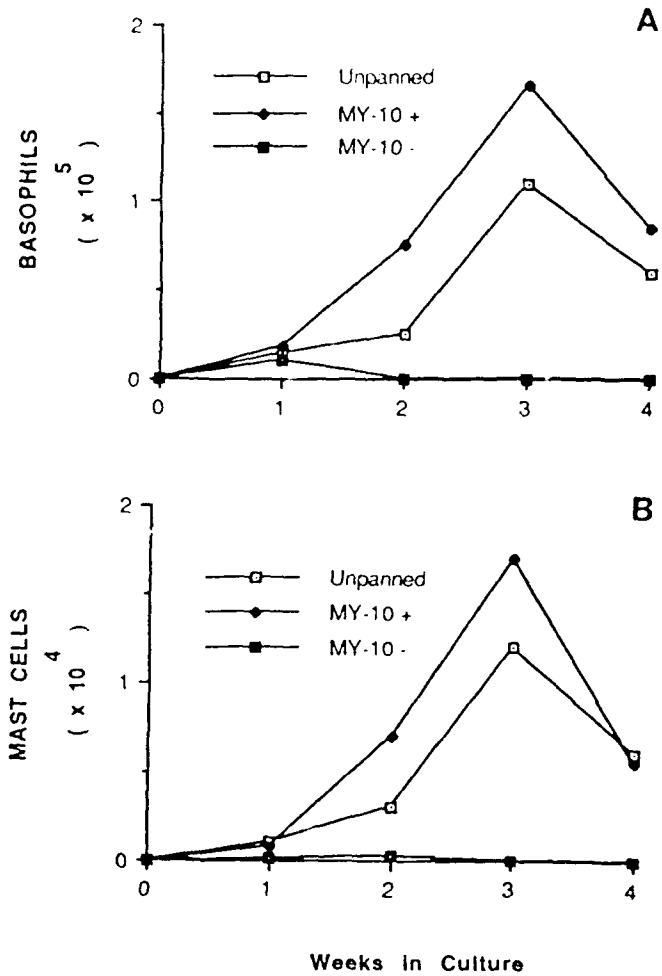

Figure 2. Growth of My-10^+ ($CD34^+$) and My-10^- ($CD34^-$) cells in the presence of rhIL-3. Basophils (*A*) and mast cells (*B*) were effectively eliminated from those cultures in which $CD34^+$ cells had been removed. Cultured My-10^- cells differentiated primarily into macrophages, with no appreciable increase in total cell numbers (data not shown). Average of two experiments.

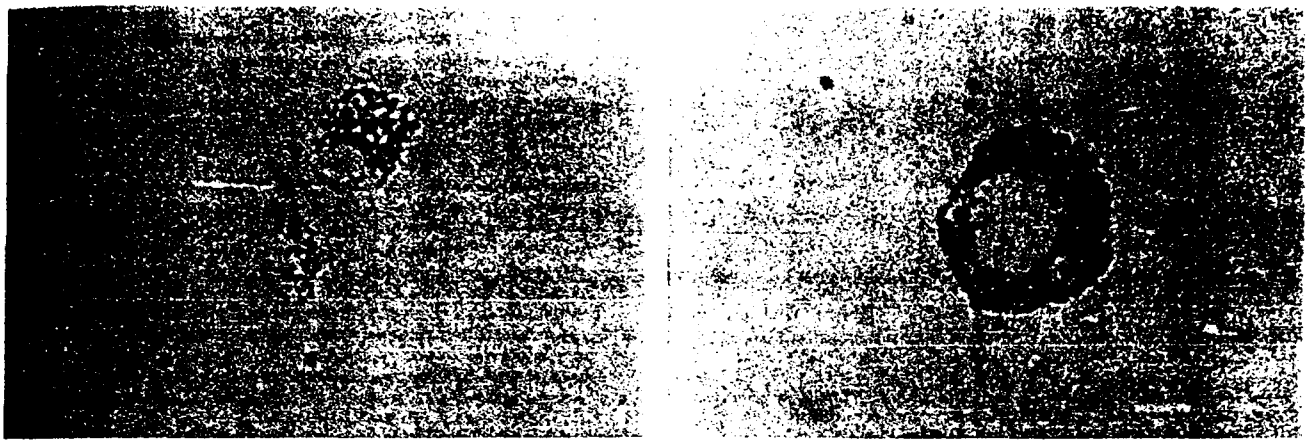

Figure 3. Light micrographs of tryptase-positive mast cells as cultured from CD34⁺ cells in the presence of rhIL-3

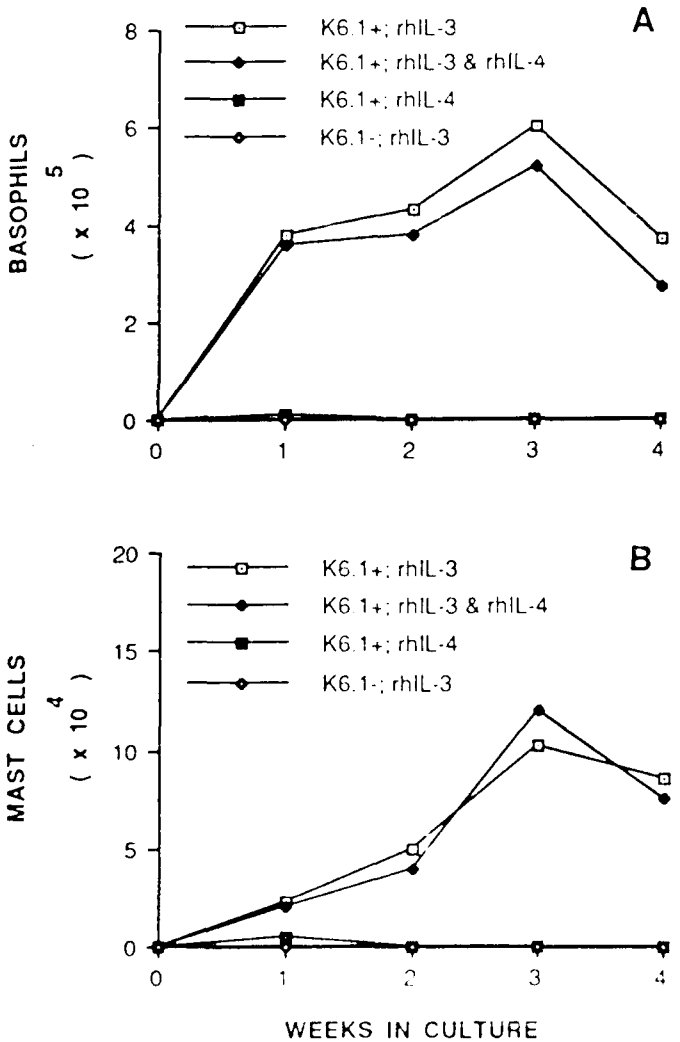

Figure 4. Growth of CD34⁺ (CD34⁺) progenitor cells in the presence of rhIL-3 alone and in combination with rhIL-4. No significant differences in the numbers of basophils (*A*) and mast cells (*B*) were noted when rhIL-3 and rhIL-4 were compared. Cultured CD34⁻ cells differentiated into macrophages with no increase in total cell numbers (data not shown). Average of two experiments.

numerous electron-dense granules (Fig. 7, *A* and *B*). Cellular surfaces stained heavily for IgE (Fig. 7, *inset*). Granules labeled for tryptase and, on ultrastructural examination, contained scroll, mixed, reticular or dense core patterns found in mature human mast cells (Fig. 8).

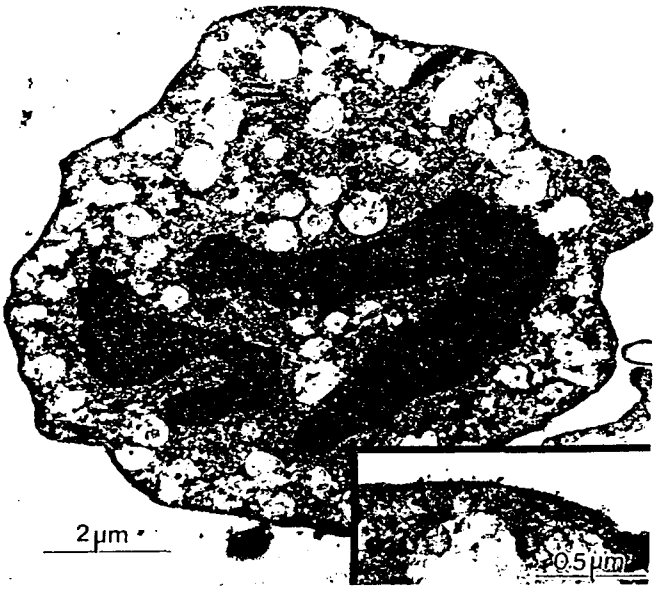

Figure 5. Electron micrograph of a basophil demonstrating 10 nm gold surface labeling for IgE but no labeling for granule associated tryptase (× 9,400) *Inset*: 10 nm gold surface labeling for IgE (× 31,900).

Human CD34⁺ progenitor cells, therefore, could also be shown to give rise to human mast cells when cocultured with 3T3 fibroblast monolayers.

DISCUSSION

In rodents, it has been demonstrated that mast cell precursors are found in bone marrow and can give rise to distinct mast cell colonies in spleen (CFU-S) when precursors are infused intravascularly in mice (1–3). These same precursors, grown in liquid suspension cultures, can give rise to mucosal-like mast cells in the presence of mouse IL-3 alone or typical connective tissue-like mast cells when precursor cells are cultured in the presence of IL-3 + IL-4 (8) or over fibroblast monolayers (5). As would be expected, mast cell precursors have been shown to originate from a common pluripotent stem cell (Thy1⁺), which can give rise to other committed progenitor cells (7). In humans, it has been speculated that mast cell progenitors also exist in the bone marrow, derive their origin from a common stem cell, and mature in the tissue under the influence of cytokines and the tissue microenvironment. Human bone marrow cells cultured

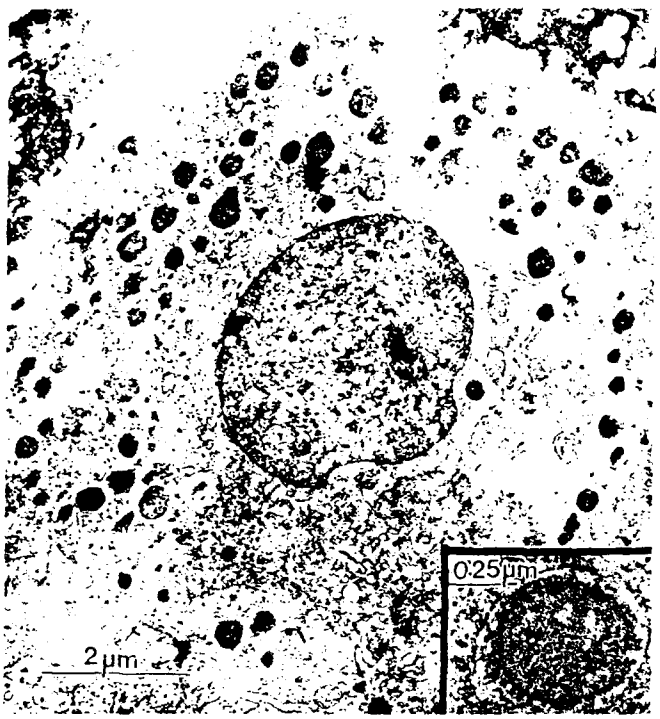

Figure 6. Electron micrograph of an interphase cultured mast cell (× 10,400) *Inset*: Mast cell granule labeled with 5 nm gold for tryptase. The granule contains homogeneously dense material (× 42,300).

in liquid suspension culture or methylcellulose have been shown to give rise to basophils (13, 16), but their ability to generate mast cells has not been examined.

More recently, it has been shown that small numbers of tryptase positive mast cells could be cultured from human bone marrow cells over agarose surfaces in the presence of rhIL-3 (10, 11). Similarly, cord blood mononuclear cells have been shown to give rise to mature mast cells when cocultured with mouse 3T3 monolayers (17). The question we asked was whether cultured human mast cells originate from pluripotent ($CD34^+$) progenitor cells found in human bone marrow or some other committed cell lineage. The bone marrow samples studied were obtained from patients under evaluation for mastocytosis. This might conceivably affect cell numbers, but not their origins. Data obtained using bone marrow cells depleted of either T cells, B cells, eosinophils, or macrophages revealed that the numbers of mast cells and basophils appearing in culture were unaffected (Fig. 1, *A* and *B*), suggesting that mast cells and basophils did not arise from committed T cell, B cell, eosinophil, or macrophage progenitor cells. Furthermore, bone marrow cells depleted of $CD34^+$ cells by the adherence (panning) method did not give rise to mast cells or basophils (Fig. 2, *A* and *B*). In contrast, highly purified collections of progenitor cells (>99% $CD34^+$) obtained using immunomagnetic selection, when cultured over agarose surfaces in the presence of rhIL-3, gave rise to large numbers of basophils and smaller numbers of tryptase positive mast cells (Fig. 5, *A* and *B*). No additional increase in cell numbers occurred when $CD34^+$ cells were cultured in the presence of rhIL-3 and rhIL-4 (Fig. 4, *A* and *B*).

Mature tissue-derived mast cells have been shown to have unique granule ultrastructure consisting of scroll, particulate, dense core, or reticular patterns (30, 31). To determine whether cultured tryptase positive mast cells

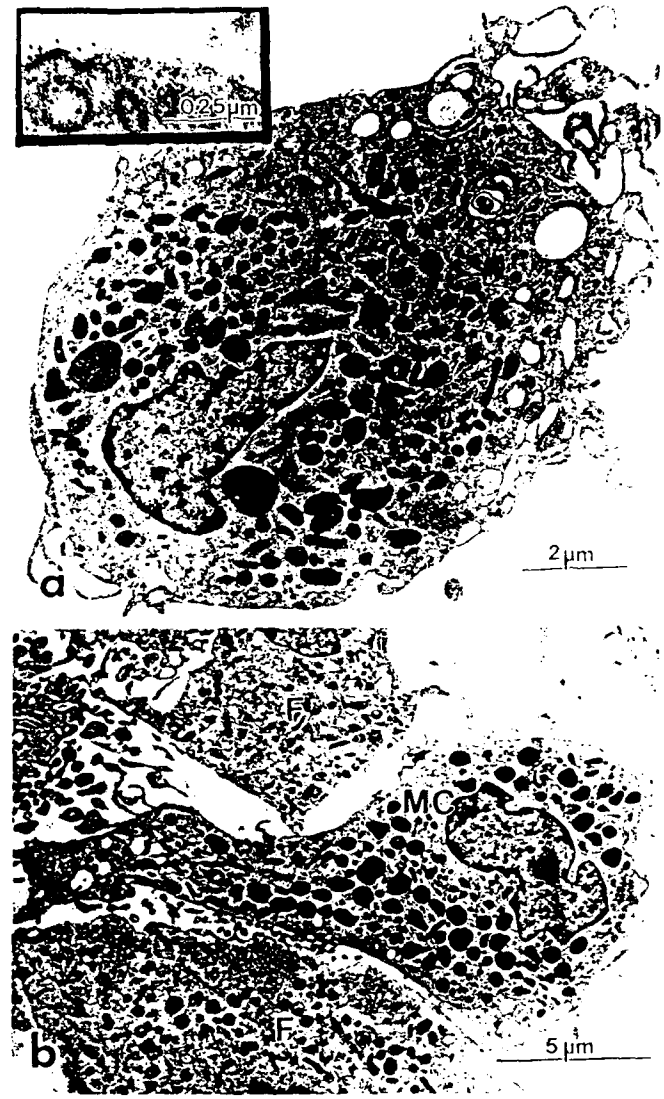

Figure 7. Electron micrographs of cocultured mast cells. (*A*, × 8,900; *B*, × 5,000) *Inset*: 10 nm gold surface labeling for IgE (× 61,200). *f*, fibroblast; *mc*, mast cell.

also contained granules with one or more of these patterns, interphase culture and 3T3 coculture derived mast cells were harvested at 3 and 6 wk, respectively, and examined using electron microscopy. Interphase cultured mast cells had granules filled with homogeneous electron-dense material similar to granules seen in immature tissue mast cells and, in some cases, mature skin mast cells (Fig. 6). Using immunogold labeling, these mast cells could be shown to have surface labeling for IgE receptors simultaneous with the demonstration of labeling over granules for tryptase (Fig. 6, *inset*). More strikingly, cocultured mast cells not only labeled for IgE and tryptase, but had a variety of granule morphologies including scroll, mixed, reticular, dense core, or homogeneous patterns found in mature human mast cells within tissues (Figs. 7, *A* and *B*, and 8).

Thus, human $CD34^+$ progenitor cells give rise to mast cells and basophils. Mast cell granule ultrastructural diversity including scroll patterns were seen only in mast cells arising from $CD34^+$ cells cocultured over mouse 3T3 fibroblast monolayers. Our data are consistent with the concept that committed mast cell progenitor cells arise in vivo in the bone marrow from $CD34^+$ pluripotent pro-

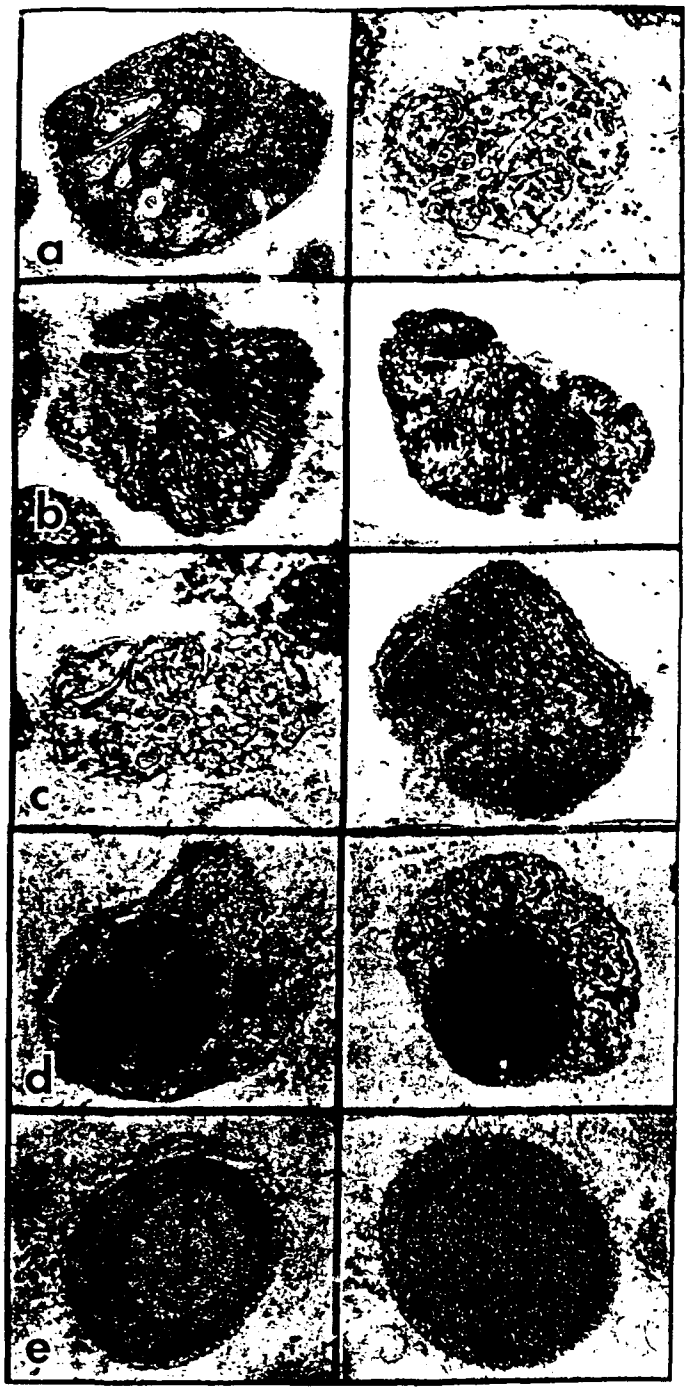

Figure 8. Electron micrographs of cocultured mast cell granules. Patterns: *A*, scroll; *B*, mixed; *C*, reticular; *D*, dense core; and *E*, mast cell granules labeled with 5 nm gold for tryptase (× 40,000)

genitor cells and mature in tissues under the influence of connective tissue cells.

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